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Postembryonic segregation of the germ line in sea urchins in relation to indirect development

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ABSTRACT **The four small micromeres of the sea urchin embryo contribute only to the coelomic sacs, which produce major components of the adult body plan during postembryonic development. To test the proposition that the small micromeres are the definitive primordial germ cell lineage of the sea urchin, we deleted their 4th cleavage parents, and raised the deleted embryos through larval life and metamorphosis to sexual maturity. Almost all of the experimental animals produced functional gametes, excluding the possibility that the germ cell lineage arises exclusively and obligatorily from descendants of the small micromeres; rather, the germ cell lineage arises during the postembryonic development of the rudiment. A survey of the literature indicates that there is no known case of an embryonic primordial germ cell lineage in a bilaterian species that displays maximal indirect development.**

Many invertebrate species produce embryonic cell lineages arising during cleavage that are the exclusive source of the germ line. Among the best known examples of precociously set aside, definitive germ cell lineages are those of *Ascaris* (1–3) and the homologous P4 lineage of *Caenorhabditis elegans* (4), and the pole cells of *Drosophila* embryos (5, 6) and many other insects (7, 8). However, autonomously specified primordial germ cell lineages have never been identified in the cleavage-stage embryos of many other relatively well known groups, such as sea urchins, ascidians, or polychaete annelids. In other groups, particularly vertebrates, definitive germ cell lineages are known to arise long after cleavage, i.e., during gastrulation. In mice, for example, primordial germ cells can first be identified in mid-primitive streak stage embryos, at day 7.25 (9). Lineages restricted to germ cell fate arise at about this time, or only shortly before, according to direct lineage labeling observations (10); earlier than this, all epiblast clones giving rise to primordial germ cells also give rise to other cell types (10–12). Definitive lineages exclusively producing germ cells are also established during gastrulation in chicken and in *Xenopus* (for review see ref. 13).

Sea urchins present an interesting case in respect to the developmental origin of the germ line. All cleavage-stage blastomeres contribute progeny to embryonic structures, except for four enigmatic cells arising in the vegetal pole in the 5th cleavage, called "small micromeres." These contribute only to the coelomic sacs (14, 15), which produce the coeloms of the adult rudiment in postembryonic development. In this report we tested the hypothesis that the small micromeres constitute a primordial germ cell lineage of the sea urchin *Strongylocentrotus purpuratus* in the same sense that the P4 lineage is the primordial germ cell lineage of *C. elegans*. Strictly defined, primordial germ cell lineages are set aside from the remainder of the embryo and are the sole possible source of the germ line, and they give rise only to germ cells. It is reasonable *prima facie* to entertain the possibility that the small micromeres could be a primordial germ cell lineage, because these cells contribute

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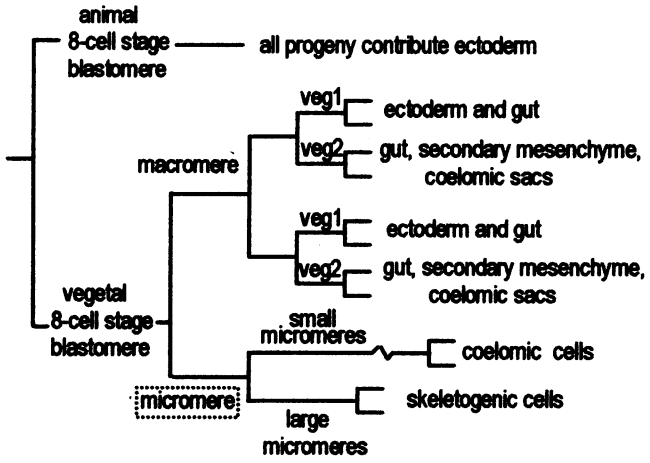

FIG. 1. Lineage of the small micromeres. The diagram depicts the progeny of one blastomere of the four-cell stage, which itself is the product of two orthogonal cleavages in the plane of the animal/vegetal axis. The four-cell stage blastomere divides equatorially to produce one animal and one vegetal daughter cell. At the eight-cell stage the embryo has four animal blastomeres, each of which contributes oral and/or aboral ectodermal progeny. The four vegetal blastomeres have identical early lineages. The small micromeres descend from the 4th cleavage micromeres by a second unequal division. The 4th cleavage micromere, representative of those deleted in the experiment described here, is enclosed by a dotted box.

to none of the differentiated tissues of the late sea urchin embryo—i.e., its gut, aboral ectoderm, oral apparatus, muscle, or nerve cells (16, 17)—nor do they play any inductive role in the signaling interactions by which blastomere specification occurs in this embryo (18, 19). The small micromeres divide only once during cleavage, and the eight cells remain passively embedded in the vegetal plate, whence they are carried inward at the tip of the archenteron during invagination. After they have been partitioned to the two coelomic sacs that arise bilaterally at the base of the esophagus, division resumes in this lineage (14). Cameron *et al.* (17) found that by the end of embryogenesis the small micromere lineage accounts for about 40% of the cells constituting the coelomic sacs. During postembryonic development these sacs give rise to the coeloms of the larval rudiment, within which the adult body plan forms.

To determine whether the small micromeres are the primordial germ cell lineage of the sea urchin we microsurgically deleted the 4th cleavage micromeres, which are the parents of the small micromeres. The deleted embryos were cultured through embryonic, larval, and juvenile stages, and when the young adults had achieved sufficient size they were tested for their ability to produce normal gametes. As shown in Fig. 1, the progeny of the four 4th cleavage micromeres are four skeletogenic lineage founder cells plus the four small micromeres. It has been known for a century (reviewed in ref. 20) that embryos lacking the 4th cleavage micromeres are capable of generating a complete larva, including a skeleton. This is due to replacement of the primary skeletogenic mesenchyme by secondary mesenchyme cells, which late in development trans-

Table 1. Survival data for embryos, larvae, and juveniles developing from embryos lacking 4th cleavage micromeres

Stage	No.	Time after fertilization
Successful 4th cleavage micromere deletions	18	5–6 hr
Pluteus stage larvae	18	3 days
Metamorphosis	17	4–8 weeks
Juveniles	15*	3 months
Young adults	7*	26 months

*Two individuals were inadvertently destroyed during transportation. Four others were lost in the initial phase of adaptation to juvenile culture. Four additional individuals died in the course of the next 18 months while in the 12°C long-term culture system.

form to skeletogenic fate if the normal skeletogenic mesenchyme is missing or depleted (21, 22). However, there are no prior reports of the postembryonic developmental potential of larvae developing from embryos lacking the 4th cleavage micromeres.

MATERIALS AND METHODS

Except for the variations indicated the procedures used in this work have all been described earlier. Micromeres were microsurgically deleted from eggs denuded of extracellular coats in low Ca^{2+} sea water, as before (23). Larvae developing from the deleted embryos were fed on *Rhodomonas* (Sp.) (24). They were cultured at progressively lower concentrations in plates containing half-filled 10-ml wells, to two larvae per well at metamorphosis. Following metamorphosis the larvae were transferred to our 12°C culture environment (25), where they were allowed to grow for about 2 years.

RESULTS AND DISCUSSION

Development of Embryos Lacking 4th Cleavage Micromeres and Generation of Normal Larvae. Eighteen partial embryos from which the 4th cleavage micromeres had been deleted were cultured. Survival at each stage of development is shown in Table 1. Remarkably, despite the major developmental role normally played by the skeletogenic micromeres, the 5th cleavage sister cells of the small micromeres, all of the 18 deleted embryos were able to complete embryogenesis and produce a more or less morphologically normal pluteus stage larva. A normal blastula and a blastula deriving from an embryo lacking micromeres, and hence devoid of the primary skeletogenic mesenchyme, are shown in Fig. 2 *A* and *B*. An example of a normal appearing late gastrula that developed from one of the experimental embryos appears in Fig. 2*C*.

Although the small micromeres take no known part in embryogenesis per se, the micromere lineages are known to have at least two different developmental functions. First, the micromeres play a role in vegetal plate specification. Ransick and Davidson (23, 26) showed that micromeres transplanted to the animal pole of a recipient embryo are capable of inducing a complete second vegetal plate, which invaginates to form a second gut. Conversely, deletion of 4th–6th cleavage micromeres from a normal embryo prevents the normal cleavage and blastula stage specification of the vegetal plate, and causes delay and in some cases abnormal morphology of gastrular invagination. Signaling from the 4th cleavage micromeres and/or their 5th–6th cleavage skeletogenic progeny (15, 23) is thus necessary for the normal early process by which vegetal plate progenitors of the archenteron are defined. A second major function of the micromere lineage is the generation of the four clones of skeletogenic mesenchyme that are founded at the 5th cleavage (see Fig. 1). In normal embryos these cells secrete the larval skeleton, which is positioned according to spatial information resident in the ectodermal blastocoel wall (27). Both of these developmental functions of the micromere lineages are executed during early development, but both can be compensated for by regulative changes in cell fate taking place much later, at or after gastrulation. The archenteron is reconstructed following a delayed invagination (26). Regeneration of the archenteron is probably a manifestation of the interactive process by which the gut develops from its progenitor cells. Thus McClay and Logan (28) have shown by microsurgical deletions that virtually any subregion of the original vegetal plate is capable of reorganizing to give rise to a complete archenteron. Similarly, as noted above, in the absence of the primary skeletogenic mesenchyme, a subset of secondary mesenchyme cells that delaminate from the tip of the archenteron acquire skeletogenic competence, and these cells proceed to create a normally formed larval skeleton (21, 22).

Table 1 shows that the secondarily organized gut and skeleton of larvae grown after micromere deletion are functionally as well as morphologically normal. Thus of the 18 plutei that completed embryogenesis, 17 successfully fed, generated complete imaginal rudiments, and underwent metamorphosis on a normal time scale. During the subsequent weeks 4 of 15 juveniles died or were lost, a rather typical score for newly metamorphosed individuals in our hands. One to 2 months later, 11 healthy juveniles were transferred to our long-term culture system (Table 1). Although a few others died over the succeeding 18 months, the majority grew at a normal rate, and after 2 years, these displayed test diameters ranging from 25 to 35 mm, a size that attests sexual maturity in normal

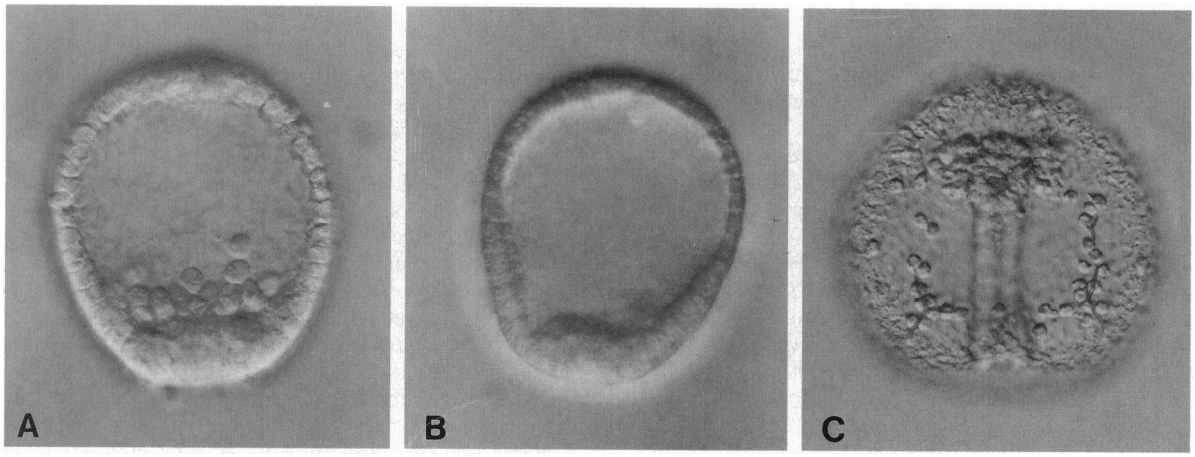

FIG. 2. Regulative development after micromere deletion. (*A*) Normal mesenchyme blastula stage embryo with primary mesenchyme cells ingressed into the blastocoel. (*B*) An equivalent stage embryo that is without primary mesenchyme after deletion of the micromeres at the 4th cleavage. (*C*) Micromere deleted embryo that has regulated to produce a normal gut and skeletogenic mesenchyme cells.

animals of this species. The overall survival rate was fairly typical for whole life cycle culture of *S. purpuratus* in our hands.

Fertility of Experimental Animals. At 26 months postfertilization, the seven surviving animals were challenged with a weak electric shock, and as Table 2 shows, all but one produced gametes. Spawning was repeated 4 months later, with the same results. The sperm produced by each of the experimental males was used to fertilize eggs from a normal female, and the eggs of the experimental females were fertilized with either the sperm of a normal male or with sperm from the experimental animals. In every case a normally developing batch of embryos was produced. The experimental animals are thus not only fertile, but their gametes function normally as well.

We conclude that the small micromeres are not in a strict sense the primordial germ cell lineage of the sea urchin. Although in normal animals their progeny may or may not contribute to the germ line, the small micromeres are clearly not the exclusive and obligatory source of the germ line, as, for example, are the pole cells of *Drosophila* or the P4 lineage of *C. elegans*. In these organisms, embryos lacking the primordial germ cell lineages produce sterile adults and no other cell lineages retain totipotency or regulate to replace missing germ cell lineages. Furthermore, we believe that our results exclude the possibility that there is any early cell lineage in the regularly developing sea urchin embryo that becomes restricted to a germ cell fate. Thus, every lineage element other than the small micromeres are known to produce progeny that give rise to a differentiated part of the embryo (irrespective of whether some of their progeny might also later give rise to germ cells).

The Larval Origin of Primordial Germ Cells in Sea Urchins. In regularly developing sea urchins such as *S. purpuratus*, the adult body plan is formed through a process of maximal indirect development. The embryo generates a feeding larva consisting of differentiated cell types, which manifests no characteristic elements of the adult body plan. The adult body plan arises from pluripotential patches of undifferentiated cells set aside from the larval structures *per se* (29). During larval life these form an imaginal rudiment, which develops by a postembryonic process different in essential aspects from that by which the embryo/larva develops. The initial population of set aside cells that can be recognized in the late embryo of regularly developing sea urchins are those constituting the coelomic sacs.

Primordial germ cells have been identified by their characteristic cytology within the left posterior coelom at metamorphosis (ref. 30; see references in ref. 31). Several months after metamorphosis they are present in the germ ring of the juveniles from which the gametes form (31). Their initial location in the larval coelom suggests that the germ cell lineages originally derive from ancestors located in the coelomic sacs of the embryo. However, the coelomic sacs are not originally prespecified prior to rudiment development. As Czihak (32) showed, complete coelomic regeneration follows destruction by UV irradiation of large portions of the coelomic sacs. Were all coelomic sac cells indeed equipotential, whether of macromere or small micromere origin, micromere deletion would not be likely to cause specific abrogation of the germ line. In the deleted embryos other cells might have altered their fates to substitute for small micromere progeny, but in respect to the germ line this again requires that exclusive competence to produce a germ line is not segregated to the micromere lineage during cleavage.

Table 2. Fertility and sex of experimental animals

Animal	Test diameter at 26 months, mm	Sex
1	24.5	—
2	27.5	♀
3	29.0	♀
4	30.0	♀
5	35.0	♂
6	35.0	♀
7	35.0	♂

Though there is no direct evidence, it is most likely that the germ cell lineages are segregated during or in consequence of the process by which different populations of cells generated in the coelomic sacs are allocated to the various regions of the adult body plan. This must occur during rudiment development. By this argument no *embryonic* primordial germ cell lineage, as specifically defined above, should exist in regularly developing sea urchins.

The Occurrence of Embryonic Primordial Germ Cell Lineages: Comparative Implication. A prediction is that *embryonic* primordial germ cell lineages should occur only in the context of direct development and never in organisms displaying maximal indirect development. Direct development, including development by means of "secondary larvae" (33), is here used to mean a process of embryogenesis in which major elements of the adult body plan emerge directly from gastrulation. This is a characteristic of all arthropods, nematodes, and chordates (see ref. 29), among other groups. Organisms that utilize maximal indirect development generate their adult body plan in specific postembryonic processes, which differ entirely from those by which the embryo produces the larva *per se*.

In Table 3, upper portion, we list the incidence of embryonic primordial germ cell lineages in bilaterian invertebrates, and in the lower portion, some examples in which the germ cell lineages do not appear until later in development. It is striking that every example of an embryonic primordial germ cell lineage indeed occurs in a directly developing organism; there is no case of such a lineage in maximally indirectly developing polychaete annelids, molluscs, or echinoderms, for example. Where its origin is known in these latter groups, the germ line is always found to arise late in embryogenesis, during development of the rudiment or its equivalent, or even later. Furthermore, the only cases in which cytoplasmic "germ cell determinants" or "germinal plasm" present in a cleavage-stage embryo have been experimentally related to germ cell lineage specification, are in direct developing organisms, e.g., frogs, *Drosophila*, or *C. elegans* (for reviews see refs. 55 and 57). Note, however, that the converse is not true: whereas some directly developing animals produce embryonic primordial germ cell lineages, others, perhaps most, define their germ line at more advanced stages, as do the terrestrial vertebrates. As indicated in Table 3, lower portion, in the cases listed in which the origin of the late appearing germ cell lineage has been ascertained, it appears to arise from mesodermal precursors, or to share lineage ancestry with mesoderm founder cells. In general, we agree with Dixon (58) that the definitive germ line segregates in the context of adult body plan formation. Though segregation may take place either early or late in that process it does not occur before that process begins.

Germ cells differ from other sets of undifferentiated pluripotential cells from which the adult body plan is formed, in both direct and indirect development. The cells that are the progenitors of the different morphological elements of the body plan are regionally assigned by signaling processes that define the anlagen of these elements in embryonic space. Germ cells may be initially marked by a state of imperviousness, or nonresponsiveness, to the signals that are used for positional specification in the various regions of the embryo and that trigger early regional patterns of transcription factor expression. It may be relevant in this connection that none of a set of genes transcribed zygotically in the early *C. elegans* embryo are expressed in the embryonic germ cell lineage until after the onset of morphogenesis (59). The impervious germ cell lineage is a modern interpretation of Weismann's original conception of the separate germ line, with the important

Table 3. Earliest appearance of germ cell lineages in bilaterian invertebrates

Genus	Taxon	Germ cell lineage	Direct (D), indirect (I)	Ref.
		Primordial germ cell lineages that segregate during cleavage		
Sagitta	Chaetognath	Founder cell segregates at fifth cleavage	D	34
Caenorhabditis	Nematode	P4 lineage segregates by 5th cleavage	D	4
Ascaris	Nematode	Germ cell lineage segregates by 5th cleavage	D	1, 3
Cyclops	Copepodan crustacean	Descendants of the 2D cell that lie near the presumptive mesoderm of the blastula	D	35
Polyphemus	Cladoceran crustacean	Descendants of the 2D cell that lie near the presumptive mesoderm of the blastula	D	36
Tetradontophora	Apterygote insect	Appear as polygonal cells from tangential cleavages at about 64-cell stage	D	37
Drosophila	Dipteran insect	Pole cells segregate during cleavage	D	6
Musca	Dipteran insect	Pole cells segregate during cleavage	D	38
Habrobracon	Hymenopteran insect	Pole cells segregate during cleavage	D	39
Calligrapha	Coleopteran insect	Pole cells segregate during cellularization of the blastoderm	D	40
Labidura	Dermapteran insect	Pole cells segregate during cellularization of the blastoderm	D	41
Liposcelis	Psocopteran insect	Pole cells segregate during cellularization of the blastoderm	D	42
		Germ cell lineages appearing in later embryogenesis or postembryonically		
Ploesoma	Rotifer	From the progeny of the D cell late in development	D	43
Lineus ruber	Nemertine worm	Primordial germ cells in the parenchyma after metamorphosis	I	44
Bonellia	Echiuran worm	In the mesoderm of the sexually undifferentiated late larva	I	45
Sphaerium	Freshwater bivalve mollusc	Arises from descendants of mesentoblasts after gastrulation	D	46
Salmacina	Polychaete annelid	In the trochophore larva, a pair of small cells descended from the M cells that descend from the 4D cell	I	47
Tubifex	Freshwater oligochaete	In the gastrula stage as the first division products of the mesoteloblasts	D	48
Hemimysis	Mysid crustacean	First seen in the gastrula in midventral area between the presumptive mesoderm and the midgut	D	49
Peripatopsis	Onychophoran	Segregate during early gastrulation as a group of cells which migrates inward with the mesoderm	D	50
Locusta	Orthopteran insect	Germ cells are first recognizable against the splanchnic mesoderm of abdominal segments	D	51
Rhodnius	Hemipteran insect	Segregated at the posterior end of the mesoderm during gastrulation	D	52
Amphipholis (-Amphiura)	Ophiuroid echinoderm	In the left somatocoel of the late larva	D	53
Asteria	Asteroid echinoderm	In the dorsal horn of the left somatocoel of the bipinnaria larva	I	54
Echinus	Echinoid echinoderm	At metamorphosis, in the left somatocoel	I	30

For reviews, extensive additional references, and widely accepted interpretations of observations regarding the origins of germ cell lineages in development see Nieuwkoop and Satasurya (57), and also Buss (56).

exception that in maximal indirect development the germ line is in fact not present and separate throughout the life cycle. In these animals the germ line arises *de novo* only after the embryo and larva have completed their own development, from the same pool of unspecified cells that give rise to the rest of the adult body.

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